

Evidence supporting a role for TopBP1 and Brd4 in the initiation but not continuation of human papillomavirus 16 E1/E2 mediated DNA replication

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Abstract

To replicate the double stranded human papillomavirus 16 (HPV16) DNA genome viral proteins E1 and E2 associate with the viral origin of replication, E2 can also regulate transcription from adjacent promoters. E2 interacts with host proteins in order to regulate both transcription and replication; TopBP1 and Brd4 are cellular proteins that interact with HPV16 E2. Previous work with E2 mutants demonstrated Brd4 requirement for the transactivation properties of E2 while TopBP1 is required for DNA replication induced by E2 from the viral origin of replication in association with E1. More recent studies have also implicated Brd4 in the regulation of DNA replication by E2 and E1. Here we demonstrate that both TopBP1 and Brd4 are present at the viral origin of replication and that interaction with E2 is required for optimal initiation of DNA replication. Both cellular proteins are present in E1-E2 containing nuclear foci and the viral origin of replication is required for the efficient formation of these foci. shRNA against either TopBP1 or Brd4 destroys the E1-E2 nuclear bodies but has no effect on E1-E2 mediated levels of DNA replication. An E2 mutation in the context of the complete HPV16 genome that compromises Brd4 interaction fails to efficiently establish episomes in primary human keratinocytes. Overall the results suggest that interactions between TopBP1 and E2 and Brd4 and E2 are required to correctly initiate DNA replication but are not required for continuing DNA replication which may be mediated by alternative processes such as rolling circle amplification and/or homologous recombination.

Importance

Human papillomavirus 16 is causative in many human cancers including cervical and head and neck and is responsible for the annual deaths of hundreds of thousands of people worldwide. The current vaccine will save lives in future generations but anti-virals targeting HPV16 are required for the alleviation of disease burden on the current, and future, generations. Targeting viral DNA replication that is mediated

by two viral proteins E1 and E2 in association with cellular proteins such as TopBP1 and Brd4 would have therapeutic benefits. This report suggests a role for these cellular proteins in the initiation of viral DNA replication by HPV16 E1-E2 but not for continuing replication. This is important if viral replication is to be effectively targeted: we need to understand the viral and cellular proteins required at each phase of viral DNA replication so that it can be effectively disrupted.

1 **Introduction**

2 Human papillomaviruses are double stranded DNA viruses that infect the epithelium and cause a variety
3 of human diseases. HPV16 is the most commonly found HPV in cervical cancer (found in around 50% of
4 cases) and also in head and neck cancer (around 90% of the HPV positive cases); see (1) for a recent
5 review. Two viral proteins, E1 and E2, are required for viral replication. E2 has a carboxyl terminus DNA
6 binding and dimerization domain that binds to 12bp palindromic sequences in the viral genome;
7 following binding the amino terminal domain of E2 can regulate transcription (2). As well as regulating
8 transcription the amino terminal domain of E2 can physically associate with E1 to recruit this protein to
9 the viral origin of replication (3, 4) whereupon E1 forms a di-hexameric complex responsible for
10 initiating and controlling DNA replication in association with a host of cellular replication factors (5-11).
11 E2 can also associate with mitotic chromatin via the amino terminal domain while simultaneously
12 binding to the viral genome; E2 therefore acts as a bridge to attach the viral genome to the host
13 chromatin during mitosis allowing efficient segregation of the viral genome into daughter cells following
14 cell division, for a review see (12). A candidate protein for mediating host chromatin attachment for
15 some E2 proteins is Brd4 (13). Co-localization of HPV16 E2 with the cellular partner protein TopBP1 at
16 mitosis suggests this protein may also play a role in segregation of the HPV16 genome (14). However,
17 Brd4 is required for the optimal transcriptional activation and repression properties of all E2 proteins
18 tested to date (15-34).

19 The initial role of E2 in DNA replication was proposed to be as an origin recognition complex that bound
20 to the viral origin of replication and then recruited the viral helicase E1 to the origin (35, 36). More
21 recently a direct role for E2 recruitment of cellular factors required for DNA replication has been
22 demonstrated. The cellular protein TopBP1, which can act as a DNA replication factor in eukaryotic cells
23 (37), is one such factor; failure of E2 to interact optimally with TopBP1 results in compromised DNA

1 replication (38). There have been conflicting reports of the role of Brd4 in regulating the DNA
2 replication of papillomavirus E2 proteins. In C33a cells HPV16 E2 mutants that have compromised
3 interaction with Brd4 have compromised replication properties (39, 40) while in CV1 cells mutations in
4 BPV1 E2 that compromise interaction with Brd4 have no effect on replication (34) although in CHO cells
5 there was compromised replication (41). In addition, nuclear foci formed by E1 and E2 have Brd4
6 present in them (39) and mutant HPV16 E2 that fails to bind Brd4 does not enter these nuclear foci (42).
7 These nuclear foci are considered to be sites of viral DNA replication therefore the presence of Brd4 in
8 these foci, and the absence of non-Brd4 interacting E2 mutants from them, implicates Brd4 as having a
9 functional role in DNA replication (39, 42-45). However, the collation of all previous results suggests
10 some ambiguity in this role and we therefore set out to investigate the role of both TopBP1 and Brd4 in
11 regulating E1-E2 DNA replication in C33a cells and also to investigate the role for TopBP1 and Brd4 in
12 maintaining the E1-E2 nuclear foci.

13 Using a real-time PCR detection method to measure E1-E2 mediated DNA replication (46) results
14 presented here suggest that both the interactions between HPV16 E2 and Brd4 and TopBP1 are
15 essential for optimum DNA replication. We also demonstrate the presence of both host proteins at the
16 viral origin of replication in an E1-E2 dependent manner using chromatin immunoprecipitation.

17 Immunofluorescence assays demonstrate that HPV16 E1 and E2 form nuclear foci that are in large part
18 dependent upon the presence of a plasmid containing the viral origin of replication suggesting these are
19 DNA replication factories. Both TopBP1 and Brd4 can be detected in these replication foci supporting a
20 role for these cellular proteins in viral DNA replication. shRNA against either TopBP1 or Brd4 completely
21 destroys the DNA replication foci induced by the expression of E1 and E2 proteins in the presence of the
22 viral origin but this does not reduce the levels of DNA replication observed. The results suggest that the
23 initiation of E1-E2 mediated DNA replication in C33a cells requires the presence of both TopBP1 and
24 Brd4 but that once initiated DNA replication progresses without the involvement of these proteins.

Experiments with an HPV16 genome containing a mutation in E2 that prevents Brd4 interaction has compromised ability to establish episomes in primary human keratinocytes, in contrast to previous experiments with the closely related HPV31 (47). This reveals a difference in the requirements of Brd4 in the life cycles of HPV16 and HPV31 and further supports the hypothesis that Brd4 is required for efficient replication of the HPV16 genome.

Materials and Methods

Cell culture and plasmids. Cells were maintained in Dulbecco's modified eagle medium (DMEM) with 10% Fetal Calf Serum (FCS) and 1% (v/v) penicillin/streptomycin mixture (Invitrogen Life Technologies) at 37 °C in a 5% CO₂/95% air atmosphere. Cells were passaged 1 in 7 every 3 days. The E2 plasmid used is described in (48) and mutant derivatives prepared as described below in mutagenesis. The pOriM plasmid used for the replication assays is described in (46) and the ptk6E2-luc plasmid is as used previously (38). The HA-E1 expression plasmid was a gift from Mart Ustav and is described in (49). The HPV18LCR was as used previously (38). pshRNA-TopBP1 has been used before by our laboratory (14) and was originally described in (50). pshRNA-Brd4 was obtained from Addgene and was originally developed by the Howley lab (17).

Mutagenesis. The R37A Brd4 mutant was prepared as described for our TopBP1 mutant (38) using the following primers. 5' GACTATTGGAAACACATGGCCCTAGAATGTGCTATTATTACAAG 3' and 5' CTTGTAATAAATAGCTCTAACTAGGGCCATGTGTTTCCAATAGTC 3'.

Western blots. Cells were harvested by trypsinization and pelleted by centrifugation. Cell pellets were washed twice with phosphate buffered saline then re-suspended in 100 µl of lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.8, 150 mM NaCl with protease inhibitor cocktail from Roche Molecular Biochemicals). The cells were lysed on ice for 30 min, and then centrifuged in a refrigerated microfuge for 10 min at 20,800 rcf at 4 °C. The supernatant was removed to a fresh 1.5 ml tube and the cell debris discarded. Protein levels were standardized using a BCA Assay (Sigma). Lysates were prepped with 10× Sample Reducing Agent (Invitrogen) and 5µl 4× LDS Buffer (Invitrogen). The lysates were run on a 4–12% gradient gel (Invitrogen) at 200 V for 1 h and transferred onto nitrocellulose membranes. Membranes were blocked in Odyssey blocking buffer (1:1 diluted with PBS) for one hour at room temperature. After blocking the membranes were incubated with 1:1000 rabbit anti HA-tag antibody to detect E1 (ab9110),

and 1:5000 (1:1000 for shRNA blot) mouse TVG261 antibody to detect HPV 16E2 (ab17185), overnight at 4°C. Membranes were washed with 0.1% PBS-tween before incubation with Odyssey secondary antibodies diluted 1:20,000 (Goat anti-mouse IRdye 800cw, 827-08364 and Goat anti-rabbit IRdye 680RD, 926-68171) for one hour at room temperature. Following secondary incubation membranes were washed 5 times for 5 minutes with 0.01% PBS-tween before infrared scanning using the Odyssey Li-Cor imaging system.

Transcription activation assay. 2×10^5 C33a cells were plated out on a 60-mm plate and transfected 24 h later using the calcium phosphate technique. The next morning cells were washed with PBS and re-fed with medium, and 24 h following the wash cells were harvested. The cell monolayers were washed twice with PBS then lysed with 300 μ l reporter lysis buffer (Promega) at room temperature for 10 minutes. Lysates were harvested by scraping and transferring into a 1.5ml microcentrifuge tube and spun in a refrigerated microfuge for 10 minutes at 4 °C with 3293rcf to clear debris. 80 μ l of the supernatant was assayed for luciferase activity using the luciferase assay system (Promega). To standardise for cell number a BCA protein assay was carried out and results expressed as luciferase units per μ g of protein.

DNA replication assay. 6×10^5 C33a cells were seeded in 100-mm² plates. The following day cells were CaPO₄ transfected with 100 pg of pOriM, 1 μ g of E1 and 10ng- 1 μ g of E2 wild-type and mutant plasmids. For shRNA replication assays the same transfection process was followed expect for the addition of 1 μ g TopBP1 and Brd4 shRNA and control shRNA (plasmids from Addgene). The following morning cells were washed twice in PBS and re-fed with medium then 48 hours later low molecular weight DNA was harvested in Hirt solution (10mMEDTA, 0.5%SDS). Samples were processed for qPCR-detected transient replication assay as previously described (46).

Chromatin Immunoprecipitation. A 100mm² dish of 60% confluent C33a cells was transfected with 1µg of pOriM, 1µg of E1 and 10ng- 100ng of E2 wild-type plasmid, using the CaPO₄ precipitation method. The following day cells were washed twice with PBS and transferred to 15cm² dishes. 48 hours post transfection cells were cross linked with 1% formaldehyde at room temperature for 15 minutes. The cross linking reaction was stopped using 0.125M glycine. Cells were harvested by scraping and then lysed in 1.5X cell pellet volume cell lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2% NP40, 10mM sodium butyrate, 50 µg/ml PMSF, 1X complete protease inhibitor). Cells were incubated on ice for 10 minutes then nuclei collected by centrifugation at 2500rpm at 4°C. Cells were then resuspended in 1.2ml of nuclear lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, 10mM sodium butyrate, 50 µg/ml PMSF, 1X complete protease inhibitor) and incubated on ice for a further 10 minutes, then diluted in 0.72ml of immunoprecipitation dilution buffer (IPDB; 20mM Tris-HCl pH 8.1, 150 mM NaCl, 2mM EDTA, 1% Triton X-100, 0.01% SDS, 10mM sodium butyrate, 50 µg/ml PMSF, 1X complete protease inhibitor). Chromatin was sonicated using a water bath sonicator (Diagenode Bioruptor 300) till chromatin was sheared to <400bp. Chromatin concentration was measured using a nanodrop and 100µg of chromatin was used per antibody experiment. The antibodies used were as followed, per IP: 2µl of a sheep anti-HPV16 E2 (amino acids 1-201) prepared and purified by Dundee Cell Products, UK; 2µg of rabbit anti-HA for detecting HA-E1 (AbCam, ab9110); 2µg rabbit anti-Brd4 (Bethyl, A301-985A1003); 2µg of mouse anti-TopBP1 (Santa Cruz, sc-271043). The antibodies and chromatin were incubated along with 20µl of a slurry of A/G magnetic beads (washed in IPDB) (Thermofisher scientific; product number 26162). Chromatin, bead and antibody slurry was incubated rotated at 4°C overnight. The following day beads were washed with 750µl IP wash buffer 1 (x2) (20 mM Tris-HCl pH 8.1, 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), then with IP wash buffer 2 (x2) (10 mM Tris-HCl pH 8.1, 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% deoxycholic acid) and TE pH 8.0 (x1 or x2). Chromatin was then prepared for qPCR by eluting the immune complexes from the beads by adding 250µl IP elution buffer (IPEB ; 100 mM

NaHCO₃, 1% SDS) and 10µg RNase A; and incubated at 65 °C for 30 mins; beads were separated from the supernatant leaving the CHIP DNA; DNA was incubated for 6 hours to overnight at 65°C. 100µg of proteinase K was added following this incubation and incubated for 2 hours at 55 °C or overnight at 45 °C. Taqman qPCR using pOriM primer and probe set was used to quantify the levels of E2, E1, TopBP1 and Brd4 at the HPV origin of replication (46).

Immunofluorescence. Cells were plated at a density of 2 X 10⁵ cells/well on acid-washed coverslips and simultaneously transfected with the indicated plasmids (Lipofectamine 2000 commercial protocol). At 24h or 48h post-transfection, cells were fixed in 4% formaldehyde and permeabilised with 0.2% Triton X-100. Cells were blocked in 1% FBS and then stained with the indicated primary and secondary antibodies. Coverslips were mounted in Vectashield mounting medium containing DAPI. Images were collected with a Zeiss LSM710 laser scanning confocal microscope and analyzed using Zen 2009 LE software.

Antibodies for immunofluorescence. The following primary antibodies were used in the immunofluorescence experiments: Ab9110 rabbit anti HA-tag [1:1000] (AbCam), HA.11 mouse anti HA-tag [1:1000] (Covance), mouse anti TopBP1 [1:100] (BD Biosciences), TVG261 mouse anti HPV16 E2 [1:10] (CR-UK), Ab128874 rabbit anti Brd4 [1:500] (AbCam). The following secondary antibodies were used in the immunofluorescence experiments: Alexa 488 donkey and rabbit [1:1000], Alexa 488 donkey anti mouse [1:1000], Alexa 555 donkey anti rabbit [1:1000], Alexa 555 donkey anti mouse [1:1000] (all Life Technologies).

shRNA. Short hairpin RNA (shRNA) against TopBP1 and the appropriate negative control were expressed from the pBABE-puro plasmid and have been previously described (50). shRNA against Brd4 was expressed from pSUPER and had the following targeting sequence: GCGGGAGCAGGAGCGAAGA (Addgene plasmid 24746) (17).

Life cycle studies. The I73A mutations in the HPV16 and HPV 31 genomes were created using the QuickChange II Site Directed Mutagenesis kit (Agilent) according to the manufacturer's directions to mutagenize the plasmid pEGFP Ni HPV16 or the plasmid pLit HPV31 with the following primers. 16E2 I73A 5' ATTGTTTCTAACGTTAGTTGCAGTTCAGcTGCTTGTAATGC 3' and 16E2 I73A 3' GCATTACAAGCAgcTGAAGTCAACTAACGTTAGAAACAAT 5'; 31E2 I73A 5' GCCAAAGCCTTACAAGCTgcTGAAGTACAAATGATGTTGG 3' and 3' CCAACATCATTGTAGTTCAgcAGCTTGTAAGGCTTTGGC 5'. Wild type and mutant genomes were transfected into human foreskin keratinocytes (HFK), selected with G418 and expanded in culture to create cell lines stably maintaining HPV16 or HPV31 as described previously (38, 47). Total DNAs from HPV16-containing cells were isolated and digested with XhoI, which does not cut the HPV16 genome, or BamHI, which cuts the HPV16 genome once. Total DNAs from HPV31-containing cells were digested with XhoI, which does not cut the HPV31 genome, or HindIII, which cuts the HPV31 genome once. Samples were analyzed by Southern blotting using the whole HPV16 or HPV31 genomes as probes.

Results

Transcriptional activation properties of wild type HPV16 E2 and non-Brd4 and non-TopBP1 interacting mutants in C33a cells

All studies in this report will describe results for HPV16 E2 unless stated otherwise therefore E2 will be used from this point. Previous work has demonstrated that an R37A mutant (arginine mutated to an alanine at position 37) in E2 compromises interaction with Brd4, this mutant will be called E2^{-Brd4} throughout the manuscript (17). Similarly, an N89YE90V mutant is compromised in TopBP1 interaction and will be called E2^{-TopBP1} (38). E2^{-Brd4} is severely compromised in transcriptional activation function (17) while E2^{-TopBP1} has altered transcriptional activation properties (38) and to confirm both mutants are behaving as predicted, transcriptional activation studies were carried out and the results are shown in Figure 1a. In lanes 1,2,3 it is clear that increasing the levels of E2^{wt} input plasmid increases the transcriptional levels (as measured by luciferase activity) from the reporter plasmid that contains 6 E2 DNA binding sites located upstream from a tk promoter. As reported previously the fold increase in transcriptional activation does not mimic the fold increase in plasmid (38). The E2^{-TopBP1} mutant is somewhat compromised in transcriptional activation at lower levels but recovers to similar levels to E2^{wt} at elevated plasmid levels, lanes 4-6, this is similar to results reported previously from 293T cells (38). E2^{-Brd4} is severely compromised in the ability to activate transcription at all levels of input plasmid, lanes 7-9, as has been reported previously by others (17). However, there remains a residual ability to activate transcription at 100ng and 1000ng input levels of plasmid with a fold increase of 2.6 and 2.4 fold respectively; this may be reflective of a residual interaction with Brd4 for this single point mutant. To confirm that the mutant proteins retain the ability to repress transcription of a HPV LCR (long control region) transcriptional assays were carried out and the results shown in Figure 1a. The levels of E2 expression plasmid used are identical as in Figure 1a. In lane 1 the HPV18LCR activity in the absence of

E2 is set as 1. It is clear that all of the E2 expression plasmids and levels repress transcription from the LCR again demonstrating that these are functional proteins. The expression levels of the E2 proteins were also determined in C33a cells following transfection of 1000ng of input plasmid DNA and the results from this experiment are shown as a western blot in Figure 1c. The upper panel is blotted for E2 and the lower panel for actin, in lane 1 when no E2 expression plasmid is added there is clearly no detectable E2 protein. In lanes 2-4 E2^{wt}, E2^{-TopBP1} and E2^{-Brd4} levels are shown respectively. There is robust expression of all of these proteins although there is more E2^{wt} than the mutants and this is observed reproducibly. Overall these results confirm that E2^{wt}, E2^{-TopBP1} and E2^{-Brd4} are expressed and behaving as predicted from previous transcription assays.

DNA replication properties of wild type and non-Brd4 and non-TopBP1 interacting HPV16 E2 mutants in C33a cells

TopBP1 is a nuclear factor involved in the initiation of DNA replication in eukaryotic systems (37) whose interaction with E2 is required for optimum DNA replication (38), Brd4 is a chromatin binding protein with a bromo-domain that can regulate the modification of histones and is involved in regulating E2 transcription function (51). The role of Brd4 in regulating E2 replication properties is less clear. A recent report demonstrated that there is a 50% drop in replication mediated by an E2-Brd4 mutant (39) while other reports suggest that Brd4 is not involved in DNA replication by E2 (41). To further investigate whether Brd4 interaction with E2 is required for E1-E2 mediated DNA replication, assays were carried out in C33a cells with E2^{wt}, E2^{-TopBP1} and E2^{-Brd4} at low and high levels of E2 input plasmid and the levels of freshly replicated DNA monitored using a sensitive real-time PCR protocol developed in our lab (46). The results are shown in Figure 2a (10ng E2) and 2b (1000ng E2); our previous work demonstrates that at 10ng E2 input level it is not possible to detect E2 mediated DNA replication by the traditional Southern blot method therefore the real-time PCR assay allows us to answer questions that cannot be addressed

by Southern blotting (46). Mock transfected, pOri only (plasmid containing viral origin of replication) and E1 expression plasmid plus pOri gives very little background signal in these assays (38, 46) therefore only the results with E2 are presented (the controls are routinely orders of magnitude lower). In Figure 2a the results are presented on a log scale and E2^{wt} 10ng levels set to 1 and the level of replication induced by mutants set relative to this. E2^{-TopBP1} and E2^{-Brd4} are all severely compromised in DNA replication with levels of replication being 0.55% and 2.57% of E2^{wt} respectively. Figure 2b describes the results obtained with 1000ng of E2 levels, our previous studies show that at this level of E2 input we would expect a recovery of the mutant replication levels compared with E2wt and this is what we observe (38). Replication levels for E2^{-TopBP1} and E2^{-Brd4} at 1000ng of input plasmid are 34.4% and 70.3% respectively when compared with E2^{wt}. This demonstrates that at higher levels of E2 the failure to initiate replication is recovered to a large degree in the mutants, perhaps not surprisingly given the elevated levels of E2 mutant proteins that could compensate for replication defects by over expression. We use a log scale in 2a and not in 2b because in 2a the levels of replication are so low that the results would be unclear on a non-log scale figure. Although these assays clearly rely on over expression of the viral proteins it can be argued that the lower levels of E2 are more reflective of what occurs in an actual infection. The levels of replication obtained with the two mutant E2 expression plasmids were very low; to determine if a double mutant would demonstrate even further reduced levels of DNA replication a double mutant was prepared that contained the R37A mutation and the N89YE90V mutation; E2^{-TopBP1/Brd4}. In both Figures 2a and 2b lane 4 it is clear that this double mutant does not further reduce the replication levels of the E2 protein. This suggests that the TopBP1 and Brd4 proteins may somehow be interacting in the same complex to regulate viral replication and that failure to bind either one of these host proteins compromises DNA replication almost to the same extent.

TopBP1 and Brd4 locate to the HPV origin of DNA replication in an E1-E2 dependent manner

The results from Figure 2 suggest that both TopBP1 and Brd4 are required for optimal DNA replication by the E1-E2 complex predicting that both proteins will be recruited to the origin of replication by E2-E1. To investigate this chromatin immunoprecipitation (ChIP) assays were carried out with chromatin prepared from C33a cells that had been transfected with a plasmid containing the HPV16 origin of replication along with E1 and E2 expression vectors incorporating relevant controls. The results are presented in Figure 3. Figure 3a shows the results obtained by carrying out the ChIP with an HA antibody that recognizes the HA-E1 protein. In lane 1 the result with non-transfected cells is shown, lane 2 has only the origin plasmid transfected while lanes 3 and 4 have the E1 and E2 expression plasmids transfected respectively; there is no difference in the signal obtained. In lane 5, when pOri plus E1 plus E2 expression plasmids are transfected then clearly there is an increase in signal of several orders of magnitude; note the results are presented on a log scale. In Figure 3b the same transfection protocol was followed through lanes 1-5 and the ChIP carried out with an E2 antibody; again a signal is only detected when pOri plus E1 plus E2 expression plasmids are transfected. This demonstrates that E2 recruitment and/or stabilization at the origin is facilitated by the presence of E1. Figure 3c shows the results of the experiment with a TopBP1 antibody where again the signal was only detectable when pOri plus E1 plus E2 expression plasmids are transfected; Figure 4b demonstrates a similar result for Brd4. There are several novel findings from these experiments: firstly, E1 and E2 co-operate to interact with the origin of replication, neither is present in the absence of the other; secondly, TopBP1 is recruited to the origin of DNA replication in an E1-E2 dependent manner; thirdly, Brd4 is recruited to the origin of DNA replication in an E1-E2 dependent manner.

TopBP1 and Brd4 are present in E1-E2 induced nuclear foci

Previous results demonstrated that E1 and E2 can locate into nuclear foci when co-expressed and some reports indicate this is enhanced by the presence of a viral origin while others suggest the presence of

origin made little difference. E2^{wt} alone or HA E1 alone, plus or minus the viral origin (pOri), could not form foci (not shown). However, in our hands, E2^{wt} plus HA E1 resulted in the formation of foci that were greatly enhanced by the presence of the origin. Figure 4a shows a typical examples of randomly selected fields from an experiment where HA E1, E2 and pOri plasmids were transfected into C33a cells. The left panel is HA staining (detecting HA E1), the middle panel E2 and the right panel a merge with a DAPI stained image. There were typically three staining patterns observed; large foci (i), punctate foci (ii) and a dispersed appearance (iii) that also looked like co-localization was occurring. To determine whether the presence of pOri influenced the distribution of these foci three independent experiments were carried out and at least 50 random images captured. The presence of the large, punctate and diffuse patterns was then counted and the results for E2 shown in Figure 4b and for HA E1 in Figure 4c; only cells that showed expression of both proteins (the large majority) were scored. Admittedly this type of quantitation is somewhat subjective (differentiating large from punctate for example) but it is clear that the presence of pOri resulted in enhanced numbers of large foci containing cells and a reduction of cells having the dispersed phenotype. This would indicate the large foci are likely replication foci. It is possible that in the absence of pOri, E1 and E2 form foci that do not increase in size as replication of pOri does not occur in these foci.

To confirm the presence of TopBP1 and Brd4 in the E1-E2-pOri foci expressing cells were stained for either TopBP1 or Brd4 and co-stained for E1 as a marker for the replication foci. Representative images are shown in Figure 5a for TopBP1 and 5b for Brd4; clearly both of these proteins locate, at least partially, into the replication foci containing E1 and E2. Both proteins in the absence of E1-E2-pOri had a more dispersed nuclear expression pattern as can be observed in the non-transfected cells shown in Figure 5a and Figure 5b.

shRNA against TopBP1 or Brd4 destroys E1-E2-pOri nuclear foci formation but has no effect on E2wt DNA replication levels.

Next we investigated whether knocking down expression of either TopBP1 or Brd4 would have an effect on nuclear foci formation and/or E1-E2 mediated DNA replication. We have used the shRNA TopBP1 plasmids previously (14) and demonstrated that it works in C33a cells and the Brd4 shRNA has been published previously (17). In Figure 6a a typical result with TopBP1 and replication foci staining is shown following co-transfection with shRNA against TopBP1 or Brd4 as indicated. It is clear in the upper left panel that in the transfected cell (evidenced by E1 in the upper middle panel) the TopBP1 levels are reduced relative to the non-transfected cells confirming that the shRNA is working. With both shRNA treatments there are no replication foci detected, only HA-E1 is stained for but E2 and the pOri plasmids were co-transfected and there is no TopBP1 detected in foci in any cell. In Figure 6b a typical result with Brd4 and replication foci staining is shown following co-transfection with shRNA against TopBP1 (i) or Brd4 (ii). Again, in the bottom left panel where E2 is being expressed (bottom middle panel) the levels of Brd4 are reduced confirming that the shRNA is working. With both shRNA treatments there are no replication foci detected, only HA-E1 is stained for but E2 and the pOri plasmids were co-transfected and there is no Brd4 detected in foci in any cell. Control shRNA plasmids had no effect on foci formation. These experiments were carried out several times with identical results and the absence of foci was observed even after 24 hours. From these results we hypothesized that E1-E2 mediated DNA replication would be affected and replication assays were carried out at various E2 plasmid concentrations; 10ng, 100ng and 1000ng in the presence and absence of shRNA plasmids targeting TopBP1 (Figure 7a) and Brd4 (Figure 7b). Neither presence of the shRNA against TopBP1/Brd4 nor the control vector had any statistically significant effect on E1-E2 DNA replication levels at all E2 levels tested; note again that both Figures have a log scale. To confirm that E1 and E2 protein levels were not affected by the shRNA treatment, western blots were carried out and the results shown in Figure 7c and 7d. At both 100ng and

1000ng levels of E2 the shRNA plasmids have no effect on either the E1 or E2 protein levels. E2 is difficult to detect at 10ng input levels.

HPV16 E2-Brd4 interaction is required for efficient establishment of episomal genomes in primary human epithelial cells

Previous work with HPV31 demonstrates that a genome encoding a mutant E2 protein that fails to interact with Brd4, I73A, has no effect on the ability of the genome to maintain episomes (47). The results presented here demonstrate that for HPV16 E2, wild type interaction with Brd4 is required for optimum DNA replication. It was therefore hypothesized that there was a difference between HPV16 and HPV31 with regards the requirement of the E2-Brd4 interaction to maintain episomal genomes and this was tested in primary human foreskin keratinocytes. A comparable mutant of HPV16 E2 that fails to interact with Brd4, I73A (17, 34), was introduced into the HPV16 genome to allow direct comparison with the HPV31 genome results (47). Wild type and mutant genomes were transfected into human foreskin keratinocytes (HFKs) and stable cell lines were cultured following drug selection. We did not observe any significant defects in the proliferation of cells containing I73A genomes as compared to wild type (not shown). Southern analysis of total DNAs from wild type cell lines are shown in Figure 8. As expected uncut DNA from HPV16 wild type cells showed a pattern of bands consistent with episomal maintenance of the genome, including a small band of supercoiled HPV DNA (upper panel lane 1). However, in cells containing I73A genomes, in the uncut lane (upper panel lane 3) all of the viral DNA was found in large, heterogeneous bands consistent with integration of HPV into the host genome, and no supercoiled band was seen even after long exposure (upper panel lane 5). When linearized, there is evidence of full length genomes being present (upper panel lanes 4 and 6) but overall there was a vastly reduced copy number when compared with the HPV16 wild type genome. This demonstrates that the I73A mutant is compromised in establishing episomal genomes demonstrating the HPV16 E2-Brd 4

1 interaction is important for establishing episomes. This was carried out with two independent mutant
2 HPV16 plasmid clones with identical results. Very similar results were obtained using three independent
3 sources of foreskin keratinocytes and a total of five experiments overall. By contrast, when the
4 analogous I73A mutation was created in the context of HPV31 and introduced into HFKs from the same
5 donors, we found that HPV31 was able to replicate episomally at copy number comparable to wild type
6 (lower panel), supporting previous results (47). These data indicate that a HPV16 E2 mutant that fails to
7 bind Brd4 cannot be maintained episomally, and that HPV16 differs from HPV31 in this respect.

8

Discussion

This report presents several novel observations that enhance our understanding of DNA replication by human papillomavirus 16 E1 and E2. Firstly, the results definitively demonstrate that a mutant of E2 that is compromised in Brd4 binding has sub-optimal DNA replication properties suggesting that Brd4 is required for efficient initiation of E1-E2 mediated DNA replication. Secondly, chromatin immunoprecipitation (ChIP) experiments demonstrate that E1 and E2 go to the viral origin of replication in a mutually dependent manner. This is novel as it has been proposed that E2 can bind to the origin in the absence of E1 but this was not observed, it is possible that E1 can stabilize the interaction of E2 with the origin allowing detection in ChIP assays, much like E1 stabilizes the E2 protein (52). Thirdly, the ChIP assays demonstrate the presence of TopBP1 and Brd4 at the viral origin of replication in an E1-E2 dependent manner. Fourthly, TopBP1 and Brd4 locate into E1-E2 foci and shRNA targeting the expression of the cellular proteins destroys these replication foci. Fifthly, E1-E2 DNA replication levels are not affected by the destruction of the E1-E2 foci therefore replication is not dependent upon these foci. Sixthly, an E2 Brd4 mutant in the context of the full length HPV16 genome fails to support efficient establishment of episomal genomes in primary human epithelial cells further supporting a role for Brd4 in mediating HPV16 genome replication in contrast to previous results with HPV31 (47).

There have been reports investigating the role of Brd4 in mediating E2 DNA replication properties, some supporting a role and some not as described in the introduction. These studies were carried out with different cell types, different E2 proteins, and different expression vectors and used different assays to monitor DNA replication. In this report C33a cells were used exclusively and the HPV16 E1 and E2 expression vectors were the same in each assay. It should also be pointed out that the replication assays that are presented in this paper are extremely sensitive and detect levels not observable on Southern blots (46). It is notable that at 1000ng of E2 expression plasmid (Figure 2b) there is very little difference

1 between the wild type E2 and E2^{-Brd4}. Therefore previous assays dependent upon the Southern blotting
2 technique may have failed to detect deficiencies of E2^{-Brd4} replication due to the limitations of the
3 technique. The failure of E2^{-Brd4} to replicate well at low levels (Figure 2a), the localization of Brd4 to the
4 origin of replication in an E1-E2 dependent manner (Figure 3d), the recruitment of Brd4 to E1-E2 nuclear
5 foci (Figure 5b), the failure of HPV16 genomes containing an E2^{-Brd4} mutant to efficiently establish
6 episomes in primary epithelial cells (Figure 8), all support a role for Brd4 in mediating HPV16 E1-E2 DNA
7 replication and having an essential role in the viral life cycle. While the mutants are slightly less well
8 expressed than wild type E2 (Figure 1c) the levels would not explain the drastic reduction in DNA
9 replication potential (0.5% and 2.5% respectively for the Brd4 and TopBP1 mutants). In addition, at very
10 low levels of E2 expression plasmid the ability to repress transcription from an HPV LCR is retained
11 (Figure 1b). Previous results with HPV31 have demonstrated that an E2^{-Brd4} mutant establishes episomes
12 as efficiently as wild type HPV31 in primary keratinocytes (47), in contrast to what is observed here for
13 HPV16. This suggests that there may be differences between the HPV types in their requirement for
14 Brd4 in genome replication and establishment. This may also explain some of the discrepancies
15 observed in prior results with different E2 proteins. The results also confirm the role for TopBP1 in
16 mediating E1-E2 DNA replication (38, 53). Previous work has shown both Brd4 (39) and TopBP1 (43)
17 recruitment to HPV replication foci further supporting a role for these proteins in this process. Knocking
18 down either TopBP1 or Brd4 had no effect on E1-E2 mediated DNA replication but completely destroyed
19 the E1-E2-pOri nuclear foci formation. One interpretation of this result is that neither TopBP1 nor Brd4
20 are required for DNA replication, but the overwhelming evidence from the results presented here and
21 by others is that this is not the case. So how can these apparently conflicting observations be reconciled
22 and do they align with previous observations? One explanation would be that an interaction between
23 both TopBP1 and Brd4 with E2 is absolutely required for efficient initiation of E1-E2 DNA replication but
24 that once initiated they are no longer required. In the shRNA experiments presented here E1 and E2

could be expressed and initiate DNA replication before the elimination of TopBP1 and Brd4. McBride and colleagues have demonstrated that Brd4 is in the heart of replication foci induced by E1-E2 but after initiation Brd4 become peripheral to the foci; this would agree with the idea that it is involved in initiation of replication but not for continuing (54). Recent work from the same group has also demonstrated association with E2 and Brd4 on similar host regions of chromatin where the viral genome replicates further suggesting a role for Brd4 in regulating E1-E2 DNA replication (55). E2 also co-localizes with TopBP1 in late mitosis and this would also associate the viral genome with host proteins that are involved in DNA replication (14). Our preliminary studies suggest that TopBP1 and Brd4 exist in the same cellular complex and therefore we propose that the TopBP1-Brd4 complex recruits E2 and the HPV genomes to locations on host chromatin that promote viral DNA replication following entry into S phase. Without this co-localization there would be a failure to properly initiate viral DNA replication perhaps due to mis-localization of the viral genomes to the “wrong” host chromatin locations. Treatment of cells with JQ1 results in a looser association of Brd4 with chromatin and slightly stimulates E1-E2 mediated DNA replication (39), this same report supports a role for Brd4 in DNA replication by E1-E2. Therefore following initiation of DNA replication, dissociation of the Brd4 containing replication complex from the host chromatin would not reduce replication if Brd4 was only required for correct initiation.

The nuclear foci formed by E1-E2-origin DNA have been proposed as DNA replication factories. This report demonstrates that both TopBP1 and Brd4 are required for the integrity of these foci as shRNA against either protein destroys their formation (Figure 5). The results also demonstrate that destruction of these foci does not affect the levels of E1-E2 DNA replication (Figure 6) suggesting that the foci induced by E1-E2 are not absolutely required for DNA replication. This is not a timing issue in the experiments presented as even after 24 hours following transfection the foci are not detected if TopBP1 or Brd4 are knocked down. DNA replication assays are harvested at least 24 hours after this and DNA replication levels increase during this time (46) therefore if foci are absolutely required for replication a

1 reduction in levels would be observed in the experiments presented. It is likely that E1-E2 replication is
2 initiated prior to the ablation of TopBP1 and Brd4. This raises the question: what are the E1-E2-origin
3 DNA foci for? There are several possible reasons for the formation of these foci that would promote the
4 viral life cycle. They may be required for the fidelity of the DNA replication or the decatenation of the
5 viral genomes following replication allowing faithful replication of viral genomes. They may be formed to
6 allow the genome to be in open chromatin locations that promote transcription of the viral genome
7 allowing expression of L1 and L2. They may be required for the encapsidation of the viral genome,
8 localizing the genomes to distinct cellular locations that will allow efficient packaging. All of these
9 options require further investigation.

10 The interpretation of these results would also require the mode of viral DNA replication to switch during
11 the assays presented. There are three different phases of replication following infection: establishment
12 that requires initiation of replication and an increase in genome copy number per cell to 20-50;
13 maintenance where the viral genome copy number per cell is kept steady during the differentiation of
14 the host cells; and finally amplification where the viral genome increases to around 1000 genome copies
15 per cell prior to encapsidation and egress. The transient transfection system presented here requires
16 initiation so therefore mimics at least one aspect of establishment. The presence of the replication foci
17 that are detected during these transient assays suggest they may also represent the amplification stage
18 of the life cycle as these foci are only ever seen in differentiated HPV genome containing cells when the
19 genome is being amplified. It has been proposed that homologous recombination is the mechanism that
20 HPV uses for amplification of the viral genome during the viral life cycle (44, 56) and the virus can
21 activate the DNA damage response to promote amplification (57). The results presented here would
22 support this model; TopBP1 and Brd4 are required for the initiation of DNA replication but would not
23 necessarily be required for a homologous recombination mechanism of DNA replication although
24 TopBP1 has been implicated in this process (58, 59).

1 In conclusion, the results presented suggest a role for TopBP1 and Brd4 in the initiation of HPV16 E1-E2
2 DNA replication but not continuing replication. The results agree with others proposing different modes
3 of viral DNA replication at different phases of the viral life cycle. Future work should focus on gaining a
4 better understanding of the cellular and viral proteins required at each stage of the viral life cycle and on
5 understanding the mechanism of replication at each phase. An added consideration is that there are
6 perhaps different requirements for DNA replication between HPV high risk types as the results
7 presented here demonstrate differences in Brd4 requirement for viral episome establishment between
8 HPV16 and HPV31. Only with a full understanding can viral replication be targeted to assist in the
9 alleviation of HPV induced disease.

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Legends

Figure 1. Transcriptional activation properties and expression levels of E2^{wt}, E2^{-TopBP1}, E2^{-Brd4}.

a) C33a cells were calcium phosphate transfected with 1µg of ptk6E2-luc and the indicated levels of E2 expression plasmid. Following cell harvest luciferase and protein assays were carried out and the results expressed as luciferase units per µg of protein. The figure represents a summary of three independent experiments carried out in duplicate; standard error bars are shown. b) C33a cells were calcium phosphate transfected with 1µg of pHPV18LCR-luc and the indicated levels of E2 expression plasmid. Following cell harvest luciferase and protein assays were carried out and the results expressed as luciferase units per µg of protein. The figure represents a summary of three independent experiments carried out in duplicate; standard error bars are shown. c) C33a cells were transfected with 1µg of each expression plasmid. Following cell harvest and protein preparation a western blot was carried out with 50µg of cellular protein extract. Following detection of E2 in the upper panel the membrane was stripped and re-probed for actin (lower panel) to ensure equivalent sample loading.

Figure 2. DNA replication properties of E2^{wt}, E2^{-TopBP1} and E2^{-Brd4} in C33a cells.

a) C33a cells were transfected with 1pg of pOri plasmid, 1000ng of HA-E1 and 10ng of each of the E2 expression vectors. Low molecular weight DNA was harvested from the cells and fresh replication assayed as described (46). Please note that this Figure uses a log scale. b) C33a cells were transfected with 1pg of pOri plasmid, 1000ng of HA-E1 and 1000ng of each of the E2 expression vectors. Low molecular weight DNA was harvested from the cells and fresh replication assayed as described (46). Please note that this Figure does not use a log scale. In both a) and b) the results represent the summary of at least three independent experiments and standard error bars are shown.

Figure 3. TopBP1 and Brd4 are recruited to the viral origin of replication in an E1-E2 dependent

manner. C33a cells were transfected with nothing (lane 1); 1µg pOri by itself (lane 2); 1µg pOri plus 1µg

HA-E1 expression plasmid (lane 3); 1 μ g pOri plus 1 μ g E2 expression plasmid (lane 4); 1 μ g pOri plus 1 μ g HA-E1 expression plasmid plus 1 μ g of E2 expression plasmid (lane 5). Following cell harvest chromatin was prepared and immunoprecipitation experiments carried out with the following antibodies a) HA (recognizes E1) b) E2 c) TopBP1 and d) Brd4. Protocols and antibody amounts are described in materials and methods. The results presented are the summary of at least three independent experiments and the standard error bars are shown. Please note that all Figures use a log scale demonstrating orders of magnitude difference between background and the positive signal in lane 5 of each Figure.

Figure 4. Localization of E1 and E2 into nuclear foci. a) C33a cells were transfected with 1 μ g pOri, 1 μ g HA E1, 1 μ g E2 and this panel shows examples of the three types of staining observed: i) Large; ii) Punctate; iii) dispersed. HA E1 or E2 by themselves did not form foci and showed a dispersed nuclear appearance (not shown). b) Cells staining positive for both E1 and E2 were scored for the types of cell staining observed for E2 in both the absence and presence of pOri. The figure represents the summary of at least three experiments with standard error bars shown. c) Cells staining positive for both E1 and E2 were scored for the types of cell staining observed for HA E1 in both the absence and presence of pOri. The figure represents the summary of at least three experiments with standard error bars shown.

Figure 5. TopBP1 and Brd4 are recruited to E1-E2 foci. a) C33a cells were transfected with 1 μ g pOri, 1 μ g HA E1, 1 μ g E2 then stained for TopBP1 (left panel) or HA (to detect HA E1, middle panel); the presence of E1 foci indicated co-expression of E2. Staining is shown in the context of DAPI staining (right panel). All cells with E1 foci demonstrated recruitment of TopBP1 to these foci, two example panels are shown. b) C33a cells were transfected with 1 μ g pOri, 1 μ g HA E1, 1 μ g E2 then stained for Brd4 (left panels) or HA (to detect HA E1, middle panels); the presence of E1 foci indicated co-expression of E2. Staining is shown in the context of DAPI staining (right panels). All cells with E1 foci demonstrated recruitment of Brd4 to

these foci, two example panels are shown. This experiment was repeated with identical results at least three times.

Figure 6. Knock down of TopBP1 and Brd4 disrupts E1-E2 DNA replication foci. a) C33a cells were transfected with 1 μ g pOri, 1 μ g HA E1, 1 μ g E2 and 1 μ g of pShTopBP1 (i) or 1 μ g pShBrd4 (ii) then stained for TopBP1 (left panels) or HA (to detect HA E1, middle panels); staining is shown in the context of DAPI staining (right panels). b) C33a cells were transfected with 1 μ g pOri, 1 μ g HA E1, 1 μ g E2 and 1 μ g of pShTopBP1 (i) or 1 μ g pShBrd4 (ii) then stained for Brd4 (left panels) or HA (to detect HA E1, middle panels); staining is shown in the context of DAPI staining (right panels). This experiment was carried out two times with identical results, the knock down of TopBP1 or Brd4 always ablated the E1-E2-pOri induced foci.

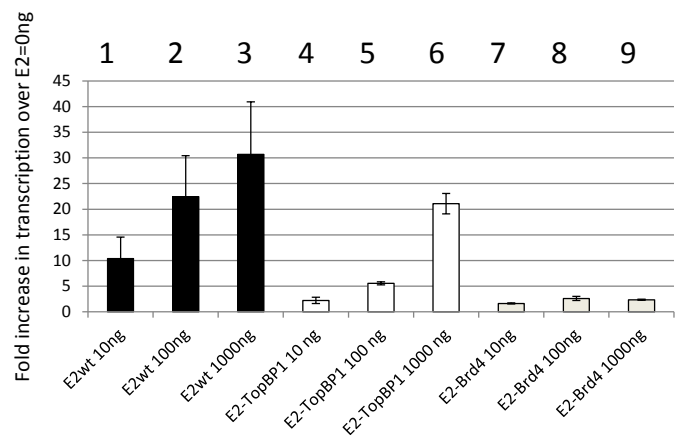
Figure 7. Knocking down TopBP1 or Brd4 as no effect on E1-E2 transient DNA replication. a) C33a cells were transfected with 1pg of pOri plasmid, 1000ng of pHA-E1 and the indicated amount of E2 expression vectors. Lanes 1,4,7 had no co-transfected plasmid; lanes 2,5,8 had 1 μ g of pshRNA control vector and lanes 3,6,9 had 1 μ g pshRNA TopBP1 (14). Low molecular weight DNA was harvested from the cells and replicated DNA assayed as described (46). b) C33a cells were transfected with 1pg of pOri plasmid, 1000ng of pHA-E1 and the indicated amount of E2 expression vectors. Lanes 1,4,7 had no co-transfected plasmid; lanes 2,5,8 had 1 μ g of pshRNA control vector and lanes 3,6,9 had 1 μ g pshRNA Brd4 (REF). Low molecular weight DNA was harvested from the cells and replicated DNA assayed as described (46). c) Where indicated in the table, C33a cells were transfected with 1 μ g of HA-E1, the indicated amount of E2 expression plasmid and 1 μ g of pshRNA Control or pshRNA TopBP1. Cells were then harvested and blotted as indicated to the left of each blot. d) Where indicated in the table, C33a cells were transfected with 1 μ g of HA-E1, the indicated amount of E2 expression plasmid and 1 μ g of pshRNA Control or pshRNA Brd4. Cells were then harvested and blotted as indicated to the left of each blot. The

DNA replication assays were carried out three times independently and the averages are shown with standard error bars.

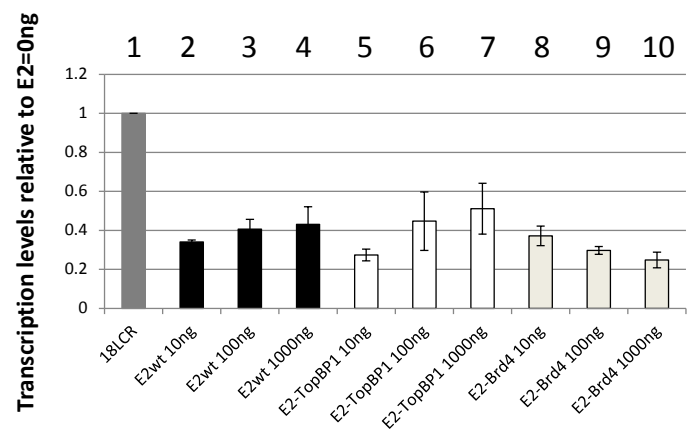
Figure 8. HPV16 genomes with defects in E2-Brd4 interaction have reduced capacity for establishing episomes. An I73A mutation was introduced into the E2 gene in the context of the entire HPV16 genome. Wild type and mutant HPV16 and HPV31 were transfected into primary human foreskin keratinocytes and the transfected cells selected using G418. Total DNAs were isolated from the cells and HPV16 samples treated with Xho1 that does not cut the genome (No cut) or BamH1 which cuts once (One cut); HPV31 samples were treated with Xho1 (No cut) and HindIII (One cut). On the upper panel there is a darker exposure of the blot shown in lanes 3,4. This result was identical in three different primary foreskin keratinocyte samples.

Figure 1.

a)



b)



c)

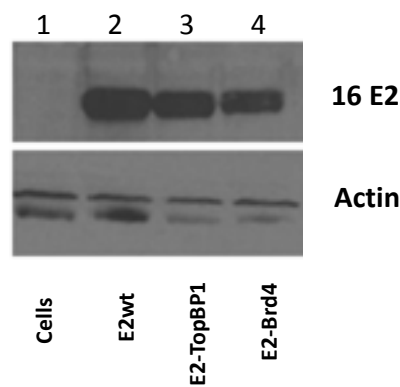
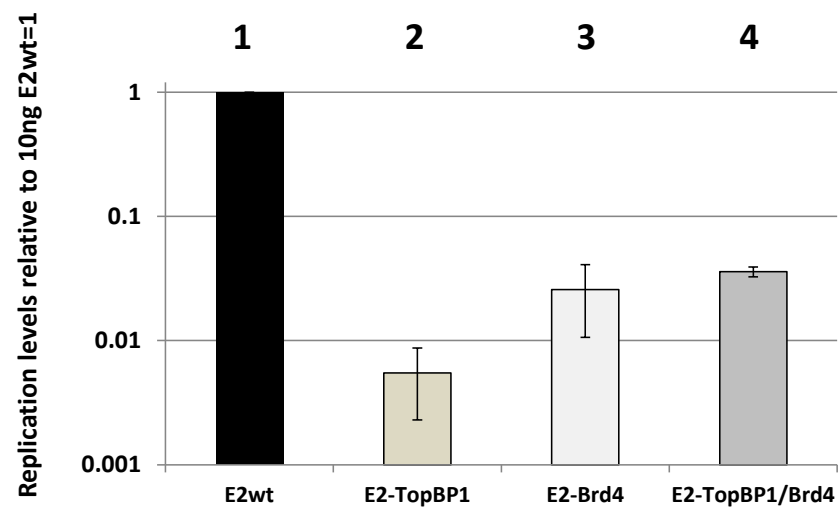


Figure 2

a)



b)

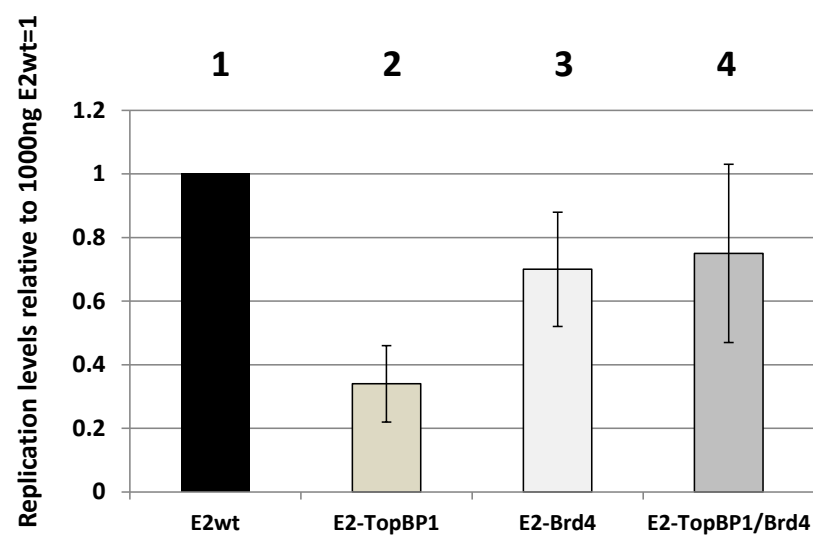


Figure 3

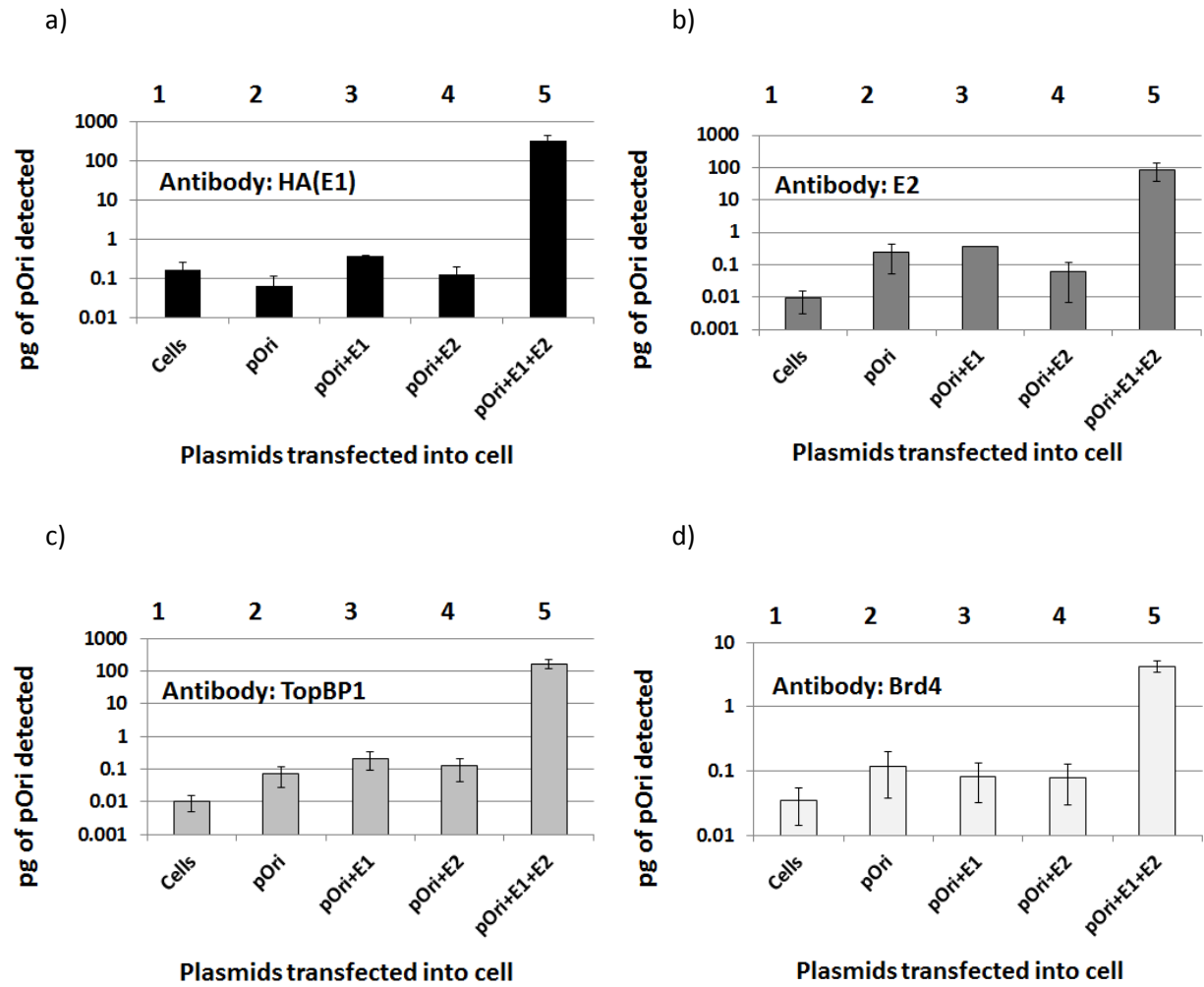
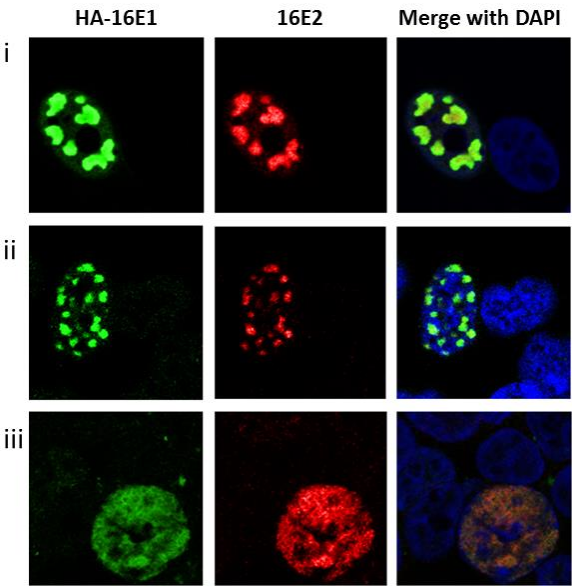
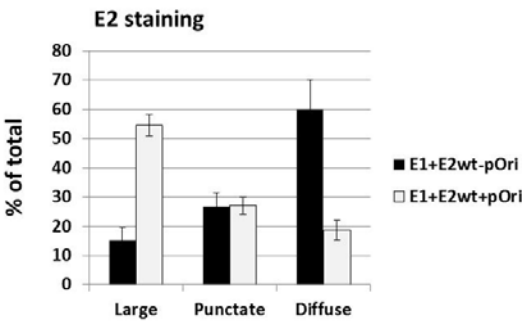


Figure 4

a)



b)



c)

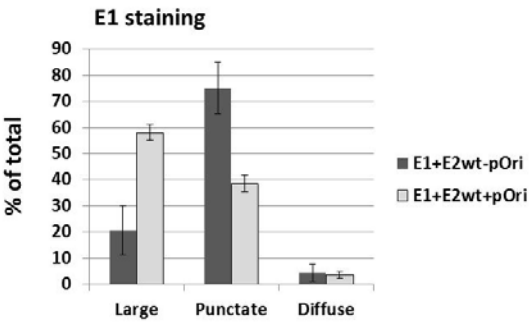
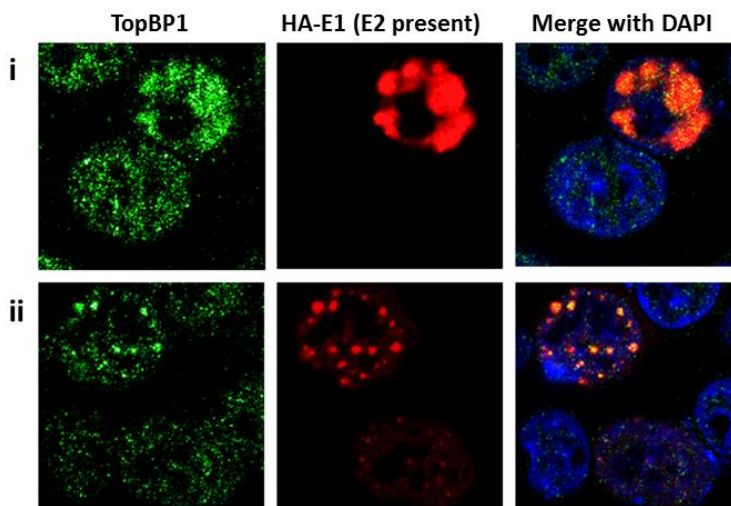


Figure 5

a)



b)

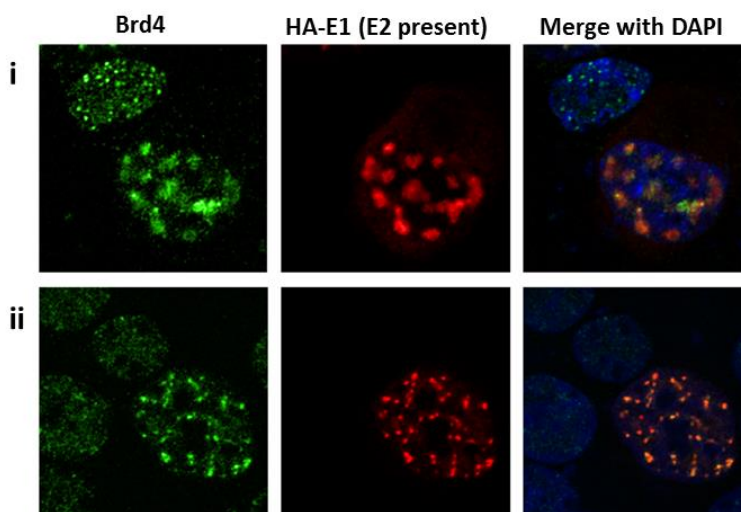
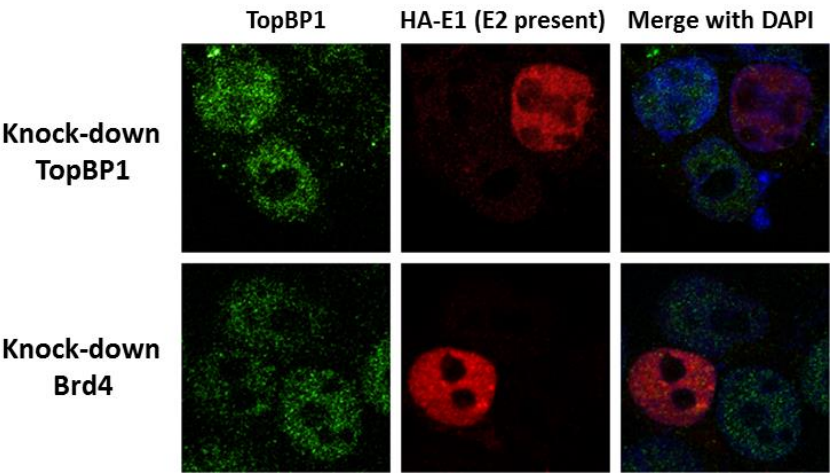


Figure 6

a)



b)

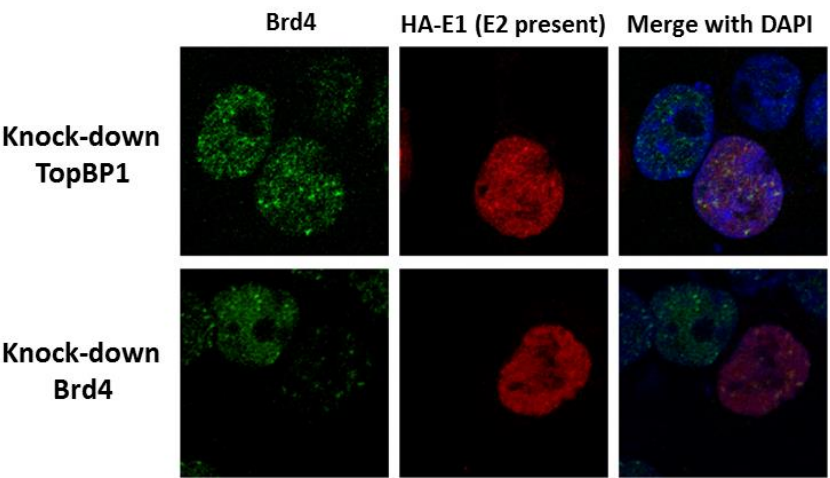


Figure 7

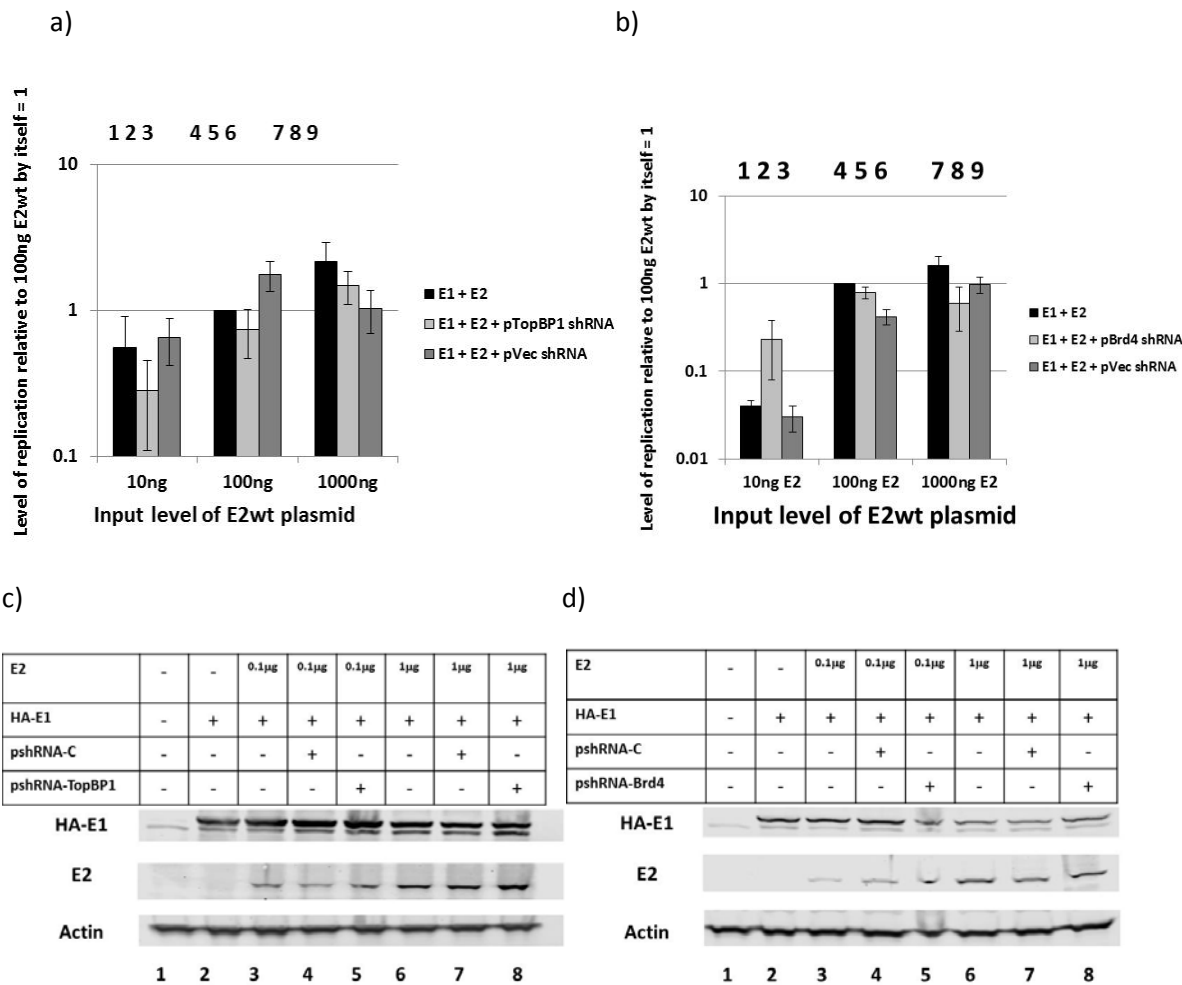


Figure 8

